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A time course study of immunological responses in *Trichuris suis* infected pigs demonstrates induction of a local type 2 response associated with worm burden

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Abstract

In order to investigate immunological changes over time in pigs infected with *Trichuris suis*, we inoculated 40 pigs with 5000 infective *T. suis* eggs and left 40 pigs as uninfected controls. Equal numbers of pigs from both groups were sacrificed every other week from 1 to 11 weeks p.i. At necropsy tissue samples were collected from all pigs and their worm burdens were determined. In the proximal colon of *T. suis*-infected pigs infiltration of eosinophils peaked 5 weeks p.i. and mast cell infiltration developed from 5 to 11 weeks p.i. Histological evaluation of the proximal colon revealed that the presence of *T. suis* was closely associated with intestinal histopathological changes such as crypt hyperplasia, goblet cell hyperplasia and a general hypertrophy of mucosa. The crypt lengths were positively associated with worm burdens. Real-time PCR analysis of genes related to immune function indicate a local increased transcription of genes coding for CCR3, ARG1, MUC5AC, IL-4, IL-5, IL-13, FcεR1α, and IL-13Rα2 and decreased expression of genes coding for iNOS, TNF-α, IL-10, CD3ε, CD80, CD86, IL-4Rα, IL-13Rα1 and CD40 in the proximal colon of pigs infected with *T. suis*. This local T-helper cell Type 2-like gene-expression pattern indicates that the Type 2 immune response characteristic of helminth infections in both mouse and humans also develops in pigs infected with *T. suis*. The results from this study expand our knowledge of the immunomodulatory effect of *T. suis*, a parasite that has proven effective in treating inflammatory bowel disease, when its eggs are administered regularly to patients.

Keywords: *Trichuris suis*; Immune response; Eosinophils; Mast cells

1. Introduction

Human trichurosis is estimated to affect more than 1 billion people worldwide (Crompton, 1999) Prevalence of infection peaks in early childhood and remains stable throughout adulthood (Bundy, 1986). Infection intensity peaks in early childhood and falls in adulthood (Bundy, 1988). Although the majority of individuals infected with the human whipworm *Trichuris trichiura* are asymptomatic, the morbidity, especially in children, is great (Stephenson et al., 2000). Chemotherapy is available but multiple treatments are required for effective removal of worms (Rossignol and Maisonneuve, 1984) and reinfections are frequent (Bundy et al., 1987). It is generally accepted that nematodes are immunogenic and several immunological responses to *T. trichiura* have been demonstrated including intestinal mast cell (Cooper et al., 1991), eosinophil and neutrophil (Kaur et al., 2002) infiltration, increased production of several Type 2 cytokines (MacDonald et al., 1994; Faulkner et al., 2002; Jackson et al., 2004a,b) and an association with high serum levels of *Trichuris*-specific antibodies (Lillywhite et al., 1991; Needham et al., 1994). Clearly there is a need to understand the immune response against this parasite with the objective of developing better protection strategies. Additionally, the immune response induced by *Trichuris* has received renewed attention as the

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use of eggs from the swine whipworm, *Trichuris suis*, has proved to be a very promising treatment for human inflammatory bowel disease (IBD) (Summers et al., 2003, 2005a,b).

The life cycle of *Trichuris* is direct. The orally ingested embryonated eggs hatch in the small intestine, the released larvae burrow into the intestinal wall of the caecum and proximal colon where they develop to mature worms (Jenkins, 1970; Beer, 1973). Protective immune mechanisms mediating worm expulsion are therefore likely to act locally but defining such local immune responses in controlled human studies is problematic. The mouse model for this disease is well established for demonstrating strain and gene-dependent acquired resistance to *Trichuris muris* infections. Although the model has provided us with a good understanding of the underlying cellular mechanisms, the effector mechanisms that culminate in worm expulsion remain largely unidentified. Strains of resistant mice mount a protective Type 2-dominated immune response resulting in worm expulsion whereas susceptible strains mount a Type 1-dominated immune response resulting in chronic infection (Else et al., 1992, 1994; Bancroft et al., 1998). However, there are several difficulties in extrapolating results obtained in a particular inbred mouse model to infections of helminth parasites in their natural animal or human host.

T. suis in pigs is, because of great similarities between the swine and human whipworms (Beer, 1976) as well as similarities between the swine and human digestive anatomy and physiology (Miller and Ullrey, 1987), considered a good model system for human trichurosis (Boes and Helwigh, 2000). Little information on immunity to *T. suis* exists but pigs do acquire protective immunity by vaccination with parasite-derived antigens (Urban, unpublished data) and by experimental inoculation with eggs (Powers et al., 1960; Roepstorff and Murrell, 1997; Pedersen and Saeed, 2001).

Age-related resistance to *T. suis* occurs in pigs (Pedersen and Saeed, 2002) similar to what is seen in human populations (Bundy et al., 1987). Increased parasite-specific antibody levels in serum (Hill et al., 1997) and severe pathology of the colon (Beer and Rutter, 1972; Rutter and Beer, 1975; Mansfield and Urban, 1996) follow infection with *T. suis*.

In the present study, we investigated the effect of a single primary *T. suis* infection on several immunological and histological parameters during the course of infection. The parasitological data from this experiment are reported elsewhere (Kringel and Roepstorff, 2006).

2. Materials and methods

2.1. Animals

Eighty crossbred Yorkshire–Danish Landrace–Duroc pigs of both sexes (39 females and 41 castrated males) were obtained from 10 different litters from a helminth-free herd. The pigs were approximately 10 weeks of age and weighed 26.8 ± 6.0 kg (mean $\pm$ SD) at the start of the experiment. During the experiment they were housed in partly slat-floored pens in groups of 8–12 pigs and were fed a standard diet consisting of ground barley plus a commercial premix (3:1) and had access to water ad libitum. The experiment was carried out with permission from the Danish animal experiments inspectorate.

2.2. Parasite infections

Infective *T. suis* eggs were originally isolated in 1993 from soil of an organic farm (Roepstorff and Murrell, 1997) and subsequently passed three times in helminth-free pigs. The eggs used for infection had been isolated from faeces of pigs, embryonated in vermiculite for 4 months according to the method described by Burden and Hammet (1976) and subsequently stored in distilled water for 18 months at 10 °C. Eggs containing viable first stage larvae were counted and experimental inoculation doses were made by re-suspension in water.

2.3. Experimental design

The pigs were divided into two groups of 40 pigs each. Allocation to groups was determined according to body weight, sex and litter by stratified randomisation. Day 0 of the experiment all pigs from one group were inoculated orally with 5000 embryonated *T. suis* eggs via a stomach tube and the pigs from the other group were kept as uninfected controls. At 1, 3, 5, 7 and 9 weeks p.i., six pigs from each group were necropsied, while the remaining 10 pigs from each group were necropsied 11 weeks p.i. Pigs were sacrificed by captive bolt pistol followed by exsanguination. Blood samples in heparin were taken from the cranial vena cava of all pigs at day 0 and from the pigs necropsied 1, 3, 5, 7 and 9 weeks p.i. again at necropsy. Of the 20 pigs necropsied 11 weeks p.i., blood samples were taken every other week from 1 to 11 weeks p.i. At necropsy approximately 100 mg of tissue from the tunica mucosa of the proximal colon (PCM) and from the colon (ileocaecal) lymph node (CLN) were collected in cryotubes, immediately snap frozen in liquid nitrogen and stored at −80 °C. Additional CLNs were collected in PBS and two full-thickness tissue samples were taken from the proximal colon from half of the control pigs and from half of the *T. suis* infected pigs. One tissue sample was fixed in 10% formalin and the other was fixed in Carnoy's solution.

2.4. Worm recovery

One, 3 and 5 weeks p.i. the immature *T. suis* were recovered from the large intestinal mucosa by incubating the intestinal pieces in 10 mM EDTA and then collecting the liberated worms on a 20 μm sieve (Kringel et al., 2002). Five (in addition to recovery by EDTA incubation), 7, 9 and 11 weeks p.i. the adult *T. suis* were recovered by washing 10% aliquots of intestinal wall washings and intestinal contents over a 212 μm sieve (Roepstorff and Murrell, 1997). For enumeration,

subsamples were counted on a gridded Petri dish. Further details in Kringel and Roepstorff (2006).

2.5. Histopathology

All of the fixed proximal colon tissue samples were processed conventionally; embedded in paraffin, sectioned transversely (2–4 μm) and mounted on glass slides. Sections from formalin fixed tissue were stained with H&E or Luna's stain for eosinophils (Luna, 1968) while the sections from tissue fixed in Carnoy's solution were stained with Toluidine Blue, pH 0.5 (Xu et al., 1993). All slides were blinded before they were assessed using a light microscope. For histopathological evaluation and measurement of crypt lengths, the H&E sections were observed at 100×/400× and 100×/250× magnification, respectively. An average of 10 crypt lengths was measured in each section with a micrometer eyepiece calibrated with a stage micrometer. All cell counts were obtained with an eyepiece grid calibrated with a stage micrometer and the average number of cells was expressed per square millimeter. In Luna sections, eosinophils in lamina propria and tela submucosa were each counted in 10 grid fields at 400× magnification covering a total tissue area of 0.625 mm^2. In Toluidine Blue sections, mast cells in the lamina propria and tela submucosa were each counted in 10 grid fields at 200× magnification covering a total tissue area of 2.5 mm^2.

2.6. Fluorescence-activated cell sorting (FACS) analysis

Peripheral blood mononuclear cells (PBMC) were isolated from heparin stabilized blood by density centrifugation using Ficoll-Hypaque and single cell suspensions from CLN were mechanically obtained by grating the lymph node on a metal grid. Cells were washed and re-suspended in PBS. A quantity of 5×10^5 isolated PBMC and CLN cells (CLNC) were incubated for 1 h at 4 °C with mouse-anti-porcine CD4a-IgG2b (clone 74-12-4), CD8a-IgG2a (clone 76-2-11), CD3-IgG2a (VMRD), CD14-IgG2b (Serotec), SWC1-IgG1 (Serotec) and SWC3(/CD172a)-IgG1/2b (VMRD) specific monoclonal antibodies in PBS +1% swine serum. Cells were thereafter washed once in PBS +1% swine serum and incubated for 1 h at 4 °C with secondary fluorescein isothiocyanate (FITC)-labeled rabbit-anti-mouse $F(ab')_2$ antibody (Dako). Finally, the cells were washed once in PBS +1% swine serum, resuspended in sheath-fluid and stored at 4 °C until FACS analysis the following day. Independent gates for lymphocytes and monocytes/macrophages were used for data collection for 10,000 events. The percentage of bound secondary antibody was quantified with the Becton Dickinson FACSCalibur flow cytometer and used as a measurement to determine the relative percentage of cells expressing the different cell surface markers: T cells ($CD3^+$, $CD4^+$, $CD8^+$, $SWC1^+$), monocytes/macrophages ($CD14^+$, $SWC1^+$, $SWC3^+$), neutrophils ($SWC1^+$, $SWC3^+$) and eosinophils ($SWC3^+$). CellQuest software (Becton Dickinson, San Jose, CA) was used to analyze data files.

2.7. RNA extraction and cDNA synthesis

RNA was extracted from PCM and CLN tissues using a commercial kit (NucleoSpin® kit, Macherey-Nagel). Approximately 30 mg frozen tissue sample was ground to a fine powder by pestle and mortar in the presence of liquid nitrogen. The frozen powder was dissolved in a lysis buffer provided in the kit and further homogenized by passing five times through a 21 G syringe needle. All RNA samples were additionally DNAse treated (DNA-*free*™, Ambion) to remove genomic DNA and the RNA concentration and integrity was measured (RNA 6000 Nano Assay, Agilent Technologies). Four point three micrograms total RNA was used for first strand cDNA synthesis using SuperScript™ II (Invitrogen, Life Technologies) and oligo(dT) for reverse-transcription PCR (RT-PCR) according to manufacturer recommendations. The cDNA was used as a template for target-specific amplification by real-time PCR to evaluate the expression of a panel of cytokine, antibody and receptor genes. All primers and Taqman probes (5′ TET- and 3′ BHQ1 labelled) were designed across adjacent exons when possible using Primer Express software (Applied Biosystems, Foster City, CA) with sequences obtained from GenBank or the TIGR porcine EST database and produced by Biosource (Camarillo CA). PCR was performed using a commercial kit (Brilliant kit, Stratagene) on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Fluorescence signals measured during amplification were processed post-amplification and were regarded as positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence (Ct-value). The relative quantification of gene transcription was normalized to the constant amount of RNA/cDNA amplified (Dawson et al., 2005). Data are presented as the mean fold change of the *T. suis* infected group related to the control group at 1, 3, 5, 7, 9 and 11 weeks p.i.

2.8. Statistical analysis

All data sets were tested for normality and variance homogeneity. Appropriate transformation was performed when necessary. The effect of *T. suis* infection and the effect of time p.i. on peripheral blood lymphocyte populations were analyzed as repeated measurements and the effect of *T. suis* infection and the effect of time p.i. on eosinophils and mast cells in the lamina propria and tela submucosa, crypt lengths and lymph node lymphocyte populations were analyzed by analysis of variance (ANOVA). The relationship between worm burdens and crypt lengths was analyzed by non-linear regression as $y=x/(a+bx)$ and the relationship between Arg1 expression in PCM and crypt length was analyzed by linear regression. Statistical significance of within tissue changes in gene expression (Ct-values) was determined by ANOVA to examine the effect of *T. suis* infection. $P<0.05$ is considered statistically significant for all analyses.

3. Results

3.1. Worm recovery peaks 3 weeks p.i.

Averages ± SD of 3328 ± 948, 5540 ± 1125, 4785 ± 2378, 4742 ± 2518, 3245 ± 3642 and 87 ± 254 worms per pig were recovered at 1, 3, 5, 7, 9 and 11 weeks p.i., respectively. Further details in Kringel and Roepstorff (2006).

3.2. Eosinophil and mast cell numbers increase in the proximal colon mucosa of T. suis-infected pigs

In the lamina propria of the proximal colon, the number of eosinophils peaked 5 weeks p.i. in pigs infected with *T. suis*. This increase is significantly ($P < 0.0001$) affected by time p.i. (Fig. 1A). The number of mast cells in the lamina propria was significantly ($P = 0.0020$) higher in the *T. suis*-infected pigs but there was no interaction with time p.i. (data not shown). In the tela submucosa of the proximal colon there were also significantly ($P < 0.0001$) more eosinophils in the *T. suis* infected pigs but there were no interactions with time p.i. (data not shown). In this tissue layer, the number of mast cells increased 5 weeks p.i. and this level remained high until 11 weeks p.i.

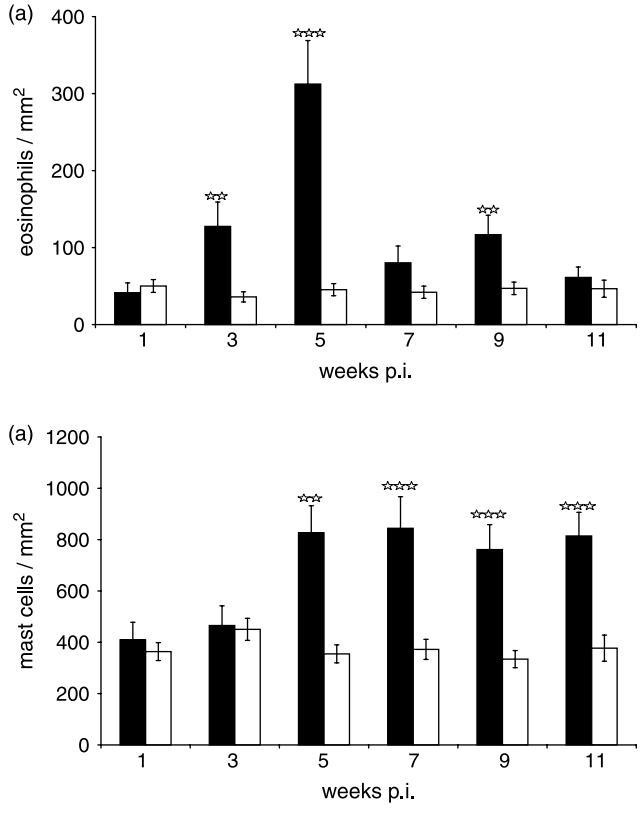

Fig. 1. (A) Eosinophils per square millimeter^2 lamina propria and (B) mast cells per square millimeter tela submucosa of the proximal colon from pigs 1, 3, 5, 7, 9 and 11 weeks p.i. with 5000 embryonated *Trichuris suis* eggs (■) and from uninfected control pigs (□). Both eosinophils in the proximal colon lamina propria and mast cells in the proximal colon tela submucosa were significantly increased over time in the pigs infected with *T. suis* ($P < 0.0001$ and $P = 0.0011$, respectively). Bars represent Least Square means ANOVA analysis ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ for *T. suis* infected pigs vs. control pigs.

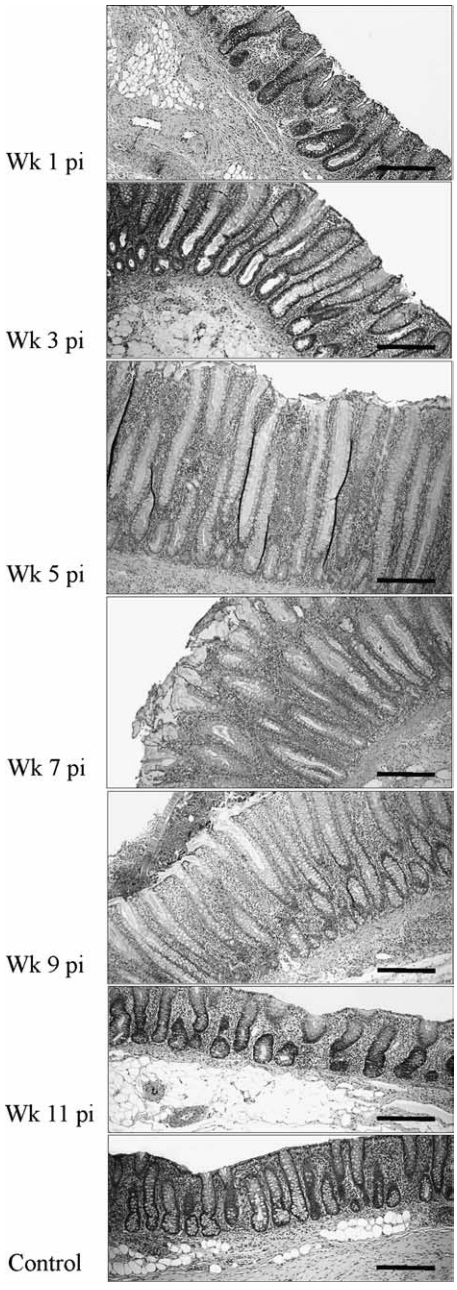

Fig. 2. Tunica mucosa of the proximal colon from pigs 1, 3, 5, 7, 9 and 11 weeks (Wk) p.i. with 5000 embryonated *T. suis* eggs and from a representative uninfected control pig. A general hypertrophy of the tunica mucosa was observed from about 3 to 9 weeks p.i. with *T. suis* compared with control pigs. H&E staining. Bar = 288 μm.

(Fig. 1B). The infection-time p.i. interaction was significant ($P = 0.0011$). From about 3 to 9 weeks p.i. histological changes were seen in the proximal colon of pigs infected with *T. suis*, resulting in infiltration of tunica mucosa and tela submucosa by inflammatory cells, crypt hyperplasia, goblet cell hyperplasia and a general hypertrophy of tunica mucosa (Fig. 2). The crypt lengths of the proximal colon were significantly ($P < 0.0001$) affected by *T. suis* infection interacting with time p.i. (Fig. 3). Furthermore, there was a significant ($P = 0.0001$) non-linear relationship between crypt lengths and worm burdens and a significant ($P < 0.0001$) linear relationship between crypt

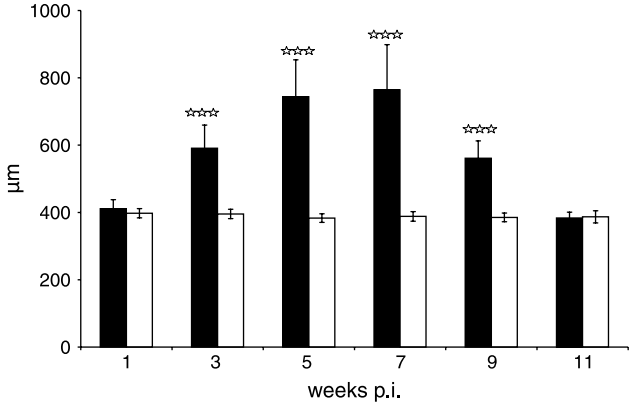

Fig. 3. Crypt lengths of the proximal colon from pigs 1, 3, 5, 7, 9 and 11 weeks p.i. with 5000 embryonated *T. suis* eggs (■) and from uninfected control pigs (□). The crypt lengths were significantly ($P<0.0001$) increased over time in the *T. suis* infected pigs. Bars represent Least Square means ANOVA analysis ±S.E.M. *$P<0.05$; **$P<0.01$; ***$P<0.001$ for *T. suis* infected pigs vs. control pigs.

lengths and Arg1 expression in PCM. The elongated crypts were dilated with mucus and a layer of mucus was present on the mucosal surface. The mucus exudates in the crypt lumen and on the surface contained neutrophils as well as degenerated epithelial cells sloughed from the mucosal surface and crypts. Neutrophils were mostly present 3 and 5 weeks p.i. and were in some cases particularly seen in the area surrounding worms. *T. suis* were macroscopically visible from 3 weeks p.i on with their anterior ends surrounded by a flat epithelial syncyticum on the luminal surface of the lamina propria.

3.3. FACS analysis reveal minor changes in cell populations of T. suis-infected pigs

No significant differences were found in the $CD3^+$, $CD4^+$, $CD8^+$ or $SWC1^+$ PBMC populations in the two experimental groups but there were significantly more $SWC3^+$ ($P<0.0001$) and $CD14^+$ ($P<0.0001$) PBMCs in the *T. suis*-infected pigs, which were not influenced by time p.i. In the CLN the $CD3^+$ and $CD8^+$ CLNC populations did not differ significantly between the two groups but the $CD4^+$ CLNC population was significantly affected by the infection-sex interaction ($P=0.0234$). Thus, infected female pigs had a lower percentage of $CD4^+$ cells whereas castrated male pigs had a higher percentage of $CD4^+$ cells compared with control pigs. There were significantly ($P=0.0164$) fewer $SWC1^+$ CLN cells in the *T. suis*-infected pigs, which was not influenced by time p.i. The number of $CD14^+$ or $SWC3^+$ cells in the CLN was below our ability to reliably detect them.

3.4. Upregulation of Th2-associated genes in T. suis-infected pigs analyzed by real-time PCR

The changes in transcription of 27 genes in PCM and CLN tissues from *T. suis*-infected pigs compared with uninfected pigs are presented in Fig. 4 (see abbreviations in Fig. 4). GATA-3 mRNA levels do not increase in PCM in pigs infected

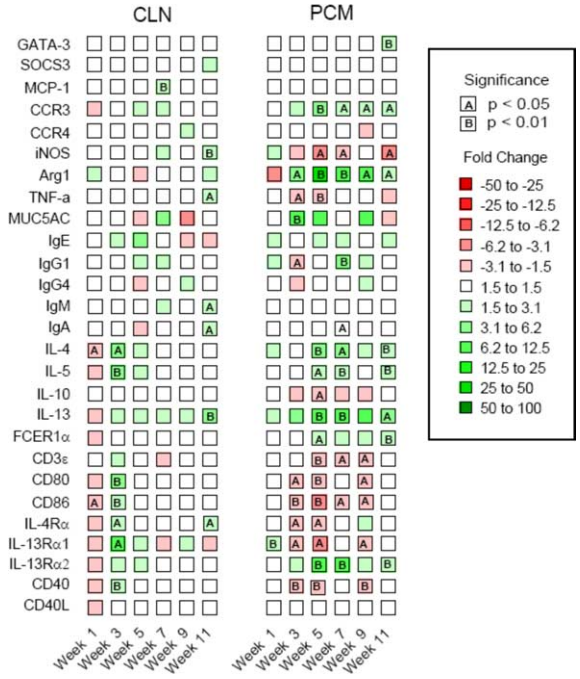

Fig. 4. Real-time PCR analysis of gene expression in colon lymph node (CLN) and the tunica mucosa of the proximal colon (PCM) of pigs 1, 3, 5, 7, 9 and 11 weeks p.i. with 5000 embryonated *T. suis* eggs compared with uninfected control pigs. A general upregulation of Type 2 associated genes were found in the *T. suis*-infected pigs compared with control pigs. The data shown represent the mean fold change in gene expression. GATA binding protein 3 (GATA-3), Suppressor of cytokine Signaling 3 (SOCS3), Monocyte chemotactic protein-1 (MCP-1), Chemokine (C-C motif) receptor 3 and 4 (CCR3 and CCR4), Inducible nitric oxide synthase (iNOS), Arginase 1 (Arg1), Tumor necrosis factor alpha (TNF-α), Mucin 5 subtypes A and C (MUC5AC), Immunoglobulin E, G1, G4, M and A heavy chain constant region (IgE, IgG1, IgG4, IgM and IgA), Interleukin-4, -5, -10 and -13 (IL-4, IL-5, IL-10 and IL-13), Fc fragment of IgE high affinity 1 receptor alpha (FcER1α), Clusters of differentiation 3 epsilon, 80, 86, 40 and 40 ligand (CD3ε, CD80, CD86, CD40 and CD40L), IL-4 receptor alpha (IL-4Rα), IL-13 receptor alpha 1 and 2 (IL-13Rα1 and IL-13Rα2).

with *T. suis* until 11 weeks p.i. and SOCS-3 mRNA levels remain unchanged in the same period of time. The monocyte chemoattractant protein (MCP)-1/CC chemokine ligand (CCL)2 mRNA level is only increased in CLN of pigs 7 weeks p.i. with *T. suis*. The chemokine receptor CCR3 mRNA expression clearly increases in PCM after *T. suis* infection whereas the chemokine receptor CCR4 mRNA expression remains unchanged in both tissues. In PCM, there is a clear inverse regulation of the two enzymes, inducible nitric oxide synthase (iNOS) and arginase type 1 (Arg1), with levels of iNOS mRNA being down regulated and Arg1 mRNA being upregulated following *T. suis* infection. Tumor necrosis factor (TNF)-α mRNA levels are downregulated in PCM 3 and 5 weeks p.i. and in the same tissue the mucin MUC5AC mRNA level is significantly elevated 3 weeks p.i. Five Ig heavy chain genes were analyzed (IgE, IgG_1, IgG_4, IgM and IgA) but few changes in the transcription of these were observed during the course of infection. Only IgG_1 was significantly downregulated 3 weeks p.i. and significantly upregulated 7 weeks p.i. in the PCM. IgM and IgA were significantly upregulated 11 weeks p.i. in the CLN. The mRNA expression of the Type 2 cytokines

IL-4, IL-5, IL-10, and IL-13, that have been associated with resistance to *Trichuris* infections in mice, are all, except for IL-10, upregulated in PCM from pigs infected with *T. suis*. In CLN there is a transient increase of IL-4 and IL-5 mRNA 3 weeks p.i. whereas the elevated level of IL-13 mRNA lasts until 11 weeks p.i. In contrast, IL-10 is down regulated in PCM during the course of infection. In the *T. suis*-infected pigs the level of the IgE high affinity receptor α-chain (FcεR1α) mRNA in PCM increases from 5 to 11 weeks p.i.. The mRNA level of CD3ε as well as of the two co-stimulatory molecules CD80 and CD86 are clearly down regulated in PCM of *T. suis* infected pigs during most of the infection. In CLN however, there is a short increase of CD80 and CD86 mRNA at 3 weeks p.i. In our study the α subunit of the IL-4 receptor, IL-4Rα mRNA levels are increased in CLN 3 and 11 weeks p.i. but decreased in PCM 3 and 5 weeks p.i. The mRNA levels of the two IL-13 binding proteins, IL-13Rα1 and -2, increase in CLN 3 weeks p.i. although IL-13Rα2 not significantly, but in PCM the mRNA levels are oppositely expressed with IL-13Rα1 being decreased and IL-13Rα2 being increased during the course of infection. The level of CD40 mRNA in CLN is increased 3 weeks p.i. but decreased in PCM, whereas the level of CD40L mRNA remains unchanged in both tissues. In summary, the expression of T helper 2 (Th2)-associated genes were increased in *T. suis* infected pigs compared with control pigs.

4. Discussion

This longitudinal study of experimental *T. suis* infections has demonstrated time p.i. dependent changes in immunological and pathological parameters that are closely associated with the presence of worms in the large intestine. The pathology is a consequence of the host immune response, which is assumed to be generated to eliminate invading pathogens. Lesions may also be caused by the direct damage from worm attachment or the indirect damage from soluble products released by the worms (Abner et al., 2002), secondary damage resulting from opportunistic bacterial infections (Mansfield and Urban, 1996) or the host's immune response to these bacteria. Neutrophils are crucial in immunity against bacteria and we did indeed observe large numbers of neutrophils in mucus exudates in *T. suis* infected pigs. In addition, the pathological changes in the proximal colon that we found associated with *T. suis* infection included infiltration of eosinophils and mast cells, crypt hyperplasia, goblet cell hyperplasia and general mucosal hypertrophy.

Infections with parasitic helminths are often associated with intestinal infiltrations of eosinophils and mast cells, observations generally considered typical for a Type 2-skewed immune response. However, several studies have failed to demonstrate the requirement of mast cells or eosinophils for protection against *T. muris* infections (Lee and Wakelin, 1982; Betts and Else, 1999; Koyama and Ito, 2000). Eosinophil recruitment is controlled by IL-5 produced by mast cells and Th2 cells, although it has been shown that eosinophilic infiltration can occur independently from IL-5 (Takamoto et al., 1997; Hogan et al., 2000). In our study IL-5 expression in the CLN increased 3 weeks p.i. and in the PCM it did not increase until 5 and 7 weeks p.i., perhaps reflecting the homing of Th2 and mast cells to the infected tissue. This time point coincides well with the peak of eosinophil infiltration in lamina propria and with the upregulation of CCR3 in the PCM. CCR3 is a receptor for several chemokines including eotaxins and is expressed by several cell types, including eosinophils (Ponath et al., 1996), basophils (Uguccioni et al., 1997), mast cells (Romagnani et al., 1999) as well as Th2 cells (Sallusto et al., 1997). Th2 cells expressing CCR3 co-localize with eosinophils at inflammed sites (Gerber et al., 1997). While the eosinophils were present in large numbers in the *T. suis*-infected mucosa, we did not see them particularly surrounding the worms as reported previously (Bundy and Cooper, 1989). Mast cells are a potent source of pro-inflammatory as well as Th2 profile cytokines (Gordon et al., 1990), though the increased numbers of eosinophils and mast cells in the infected tunica mucosa may represent an immuno-pathological rather than a protective response. Granulocytes may also play a role in the induction or maintenance of the Th2 response. IL-4, in synergy with stem cell factor (SCF), regulates human intestinal mast cell proliferation and enhance mediator release (Bischoff et al., 1999) and it further enhances the expression of Th2 cytokines (IL-3, IL-5, IL-9, and IL-13) by mast cells activated by IgE receptor cross-linking (Lorentz et al., 2000). In mice the high affinity IgE receptor (FcεR1) is expressed only by mast cells and basophils whereas the expression of FcεR1 in humans extends to other cells types as well (Kinet, 1999). The increased number of mast cells in the tela submucosa of *T. suis*-infected pigs from 5 to 11 weeks p.i. coincides with the increase in FcεR1α mRNA in PCM.

It is generally accepted that the IL-4/IL-13 system plays an essential role in the resistance response against *T. muris* (Bancroft et al., 1998; 2000) but the effector mechanisms whereby IL-4 and IL-13 mediate worm expulsion is unknown. However, there is growing evidence that they exert a number of effects on mucosal epithelium (Shea-Donohue et al., 2001; Madden et al., 2004) that in mice may involve TNF-α (Artis et al., 1999a). It was suggested that TNF-α plays a role in regulating Th2 effector activity by regulating IL-4 and IL-13 receptor expression on intestinal cells, a function that has been observed in human endothelial (Lugli et al., 1997) and fibroblast (Yoshikawa et al., 2003) cells. IL-4 and IL-13 have a number of overlapping functions, owing in part to their sharing of a receptor, the IL-4Rα chain. The concurrent decrease in TNF-α, IL-4Rα, and IL-13Rα1 mRNA levels 3 and 5 weeks after *T. suis* infection and the concurrent increase in IL-4, IL-13, and IL-13Rα2 mRNA levels 5–11 weeks p.i. could indicate a tightly controlled reduction in IL-13 signaling capacity.

TNF-α is known to be a key mediator of pathogenesis in a broad range of infectious and predominantly Th1-mediated inflammatory diseases, possibly via macrophage iNOS induction and thus NO synthesis (Corradin et al., 1991). However, NO has also been shown to enhance the induction of TNF-α synthesis (Huang et al., 1998). Several studies have found a positive correlation between iNOS activity levels and

disease severity in patients with ulcerative colitis, while a similar association is less clear in Crohn's disease (Kimura et al., 1997; Guihot et al., 2000). In our experiment *T. suis* significantly downregulated iNOS expression and upregulated Arg1 expression in the PCM, an observation that is in agreement with the clinical improvement of IBD patients receiving *T. suis* eggs (Summers et al., 2003, 2005a,b) and that is likely to represent the presence of alternatively activated macrophages (Gordon, 2003), a type of macrophage presumably important in the Type 2 response against parasitic helminths. They may function to moderate the inflammatory response to the parasite and as effector cells releasing anti-parasite molecules and they may be important for wound repair (Maizels et al., 2004). The two enzymes iNOS and Arg1 share L-arginine as a common substrate, where Arg1 converts L-arginine to L-ornithine that is further used for producing polyamines that can stimulate colonic epithelial cell growth (Ray et al., 2001). In the *T. suis*-infected pigs we found a significant positive linear relationship between Arg1 expression and crypt lengths of the PCM. The potential importance of arginase in intestinal inflammation may be twofold; modulation of NO production by competitive inhibition of iNOS (Chang et al., 1998) and production of polyamines, which themselves may modulate intestinal pathophysiology. Macrophage Arg1 expression and increased concentration of circulating polyamines have been reported in *Schistosoma mansoni* infections (Abdallahi et al., 2001). Resistance to *T. muris* is associated with a striking increase in epithelial cell proliferation in the infected caecum (Artis et al., 1999b) and a recent study demonstrates that IL-13 dependent epithelial cell turnover is an effective expulsion mechanism of *T. muris* (Cliffe et al., 2005). Consistent with this report, the dramatic elevation of the proximal colon crypt lengths duing *T. suis* infection is closely associated with the size of the worm burden. Thus, with worms present in the colon, an immune regulated elevation in epithelial cell proliferation and crypt cell hyperplasia could result in the detachment of worms from the epithelium.

Increased number of goblet cells, upregulated mucin secretion and qualitative changes in mucus secretion and composition follow infection with a number of nematode parasites but are rarely reported from studies on *T. suis*. MUC5AC is a tracheobronchial/gastric mucin that is not normally expressed at high levels in colonic tissue. It is expressed in the colon mucosa of *T. suis*-infected pigs as well as in goblet cells in IBD and other inflammatory conditions of the colon (Shaoul et al., 2004). Mucins may, though, be functioning in the repair of the damaged intestinal epithelium or possibly in the initial nonspecific response to pathogens. The toll like receptors (TLR) is a group of pattern recognition receptors and the importance of these in the mammalian host as well as the presence of molecular patterns expressed by helminth parasites is still largely unknown but TLR4, expressed by antigen-presenting cells, has proved to be crucially involved in the response against *T. muris* (Helmby and Grencis, 2003). In the peripheral blood of *T. suis*-infected pigs, we found an increased percentage of cells expressing CD14, the co-receptor for TLR4. Higher percentages of CD14 as well as SWC3 positive PBMC in pigs infected with *T. suis* could indicate increased numbers of monocytes/macrophages migrating to the site of infection.

MCP-1/CCL2 is chemotactic for various cell types, including monocytes/macrophages, activated T cells and basophils and was only temporarily upregulated in the CLN of *T. suis*-infected pigs. It has been shown to be associated with a Th2-dominated response and necessary for developing resistance against *T. muris* (deSchoolmeester et al., 2003). Very little has been published on the role of chemokines in parasitic infections although they have functions in developing Th1/Th2 immunity beside their functions in chemotaxis.

T cell interaction with the co-stimulatory molecules (CD80 and CD86) expressed by antigen presenting cells (APC) is required for Th cell effector functions and it has been demonstrated that this co-stimulation is required to induce IL-4 but not IL-13 responses in *T. muris* infections (Urban et al., 2000). The brief upregulation of CD40, CD80, CD86 and CD3ε mRNA in CLN 3 weeks p.i. and the simultaneously decreased levels of CD80, CD86 and IL-10 mRNA in PCM, suggest emigration of *T. suis* antigen-stimulated APC (Dendritic cells and B cells) from the tunica mucosa to the lymph nodes where they direct the development of Th2 cells. CD40 and CD40L are involved in B cell activation interactions between B and T cells and they are expressed on B and T cells, respectively (Armitage et al., 1992). CD40 is also found on all accessory cells, i.e. macrophages and other nonlymphoid cells. The involvement of dendritic cells in protective Th2 responses during the course of *T. muris* infection has been reported (Koyama, 2005) and a lower number of plasma cells in proximal colon lamina propria during *T. suis* infection were observed in the histological evaluations (data not shown). The low expressions of Ig genes, in particular IgE and IgA, in *T. suis*-infected pigs are surprising, as antibody responses generally are expected in helminth infections. However, most reports on parasite-specific antibody responses are measured in serum (Scaglia et al., 1979; Needham et al., 1994; Faulkner et al., 2002) and it is commonly known that each body fluid has a particular distribution of Ig isotypes. Also, one must bear in mind that in this study we used gene expression analysis and not parasite specific antibody analysis. Swine IgG_1 is hypothetically a Type 2 isotype (Furesz et al., 1998) and a brief upregulation of this gene in the PCM 7 weeks p.i. corresponds to the overall Type 2 response presumably being induced. IgG_4 belongs to a different cluster than IgG_1 and information on this isotype expression and protein is lacking. Unlike dendritic cells, the role of B cells as cytokine producing cells involved in the priming of $CD4^+$ cells is not well described, but a sub-population of B cells along with dendritic cells produce IL-10, a cytokine that has been identified as a critical component of the resistance mechanism in mice by counter regulating the Th1 cytokine response (Schopf et al., 2002). This cytokine, along with transforming growth factor (TGF)-β, is also produced by T cell subpopulations that have been termed Regulatory T cells. Their function in infectious disease immunology has received recent attention (Belkaid

et al., 2002; Kullberg et al., 2002) but what role IL-10 plays in *T. suis* immunity is not clear. *T. suis* excretory/secretory products elicit IL-10 secretion from intestinal epithelial cells within 24 h, suggesting the involvement of this cytokine in the very early host response creating a Th2-microenvironment (Parthasarathy and Mansfield, 2005).

The current hypothesis of human immunity to trichuriasis was developed primarily in the mouse model (deSchoolmeester and Else, 2002; Else and deSchoolmeester, 2003) and outbred pigs apparently develop a Type 2-dominated immune response against *T. suis* similar to that of *T. muris* resistant mouse strains. In the infected resistant mouse, the combination of pattern recognition receptors is likely to determine the ability of the early innate immune response to create a cytokine mileu that promotes Th2 polarization within the draining lymph nodes. These $CD4^+$ Th2 cells further promote the Th2 response by producing IL-4, IL-5, IL-10 and IL-13 and along with other effector cells such as eosinophils and macrophages, they get recruited to the site of infection by intestinal epithelial cell produced chemokines. Cytokine induced changes in the intestinal epithelium that increase epithelial cell turnover may represent an effector mechanism resulting in worm expulsion. However, further research in pigs is needed to fully determine homologies and deviations from the hypothesis described above, as well as the significance of granulocytes and mast cells in the early response and the role of the alternatively activated macrophages and regulatory T cells. The apparent agreement between the immune responses elicited by *T. suis* and *T. muris* leads us to consider the swine model a good supplement to the mouse model for understanding *Trichuris* infections of humans as outbred large animals more closely mimic the human population.

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Supplementary data

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